Chapter 1

Introduction and scope of the thesis

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Introduction

A fundamental property of eukaryotic cells is the ability to compartmentalize biochemical processes into specific areas within the cell. Cells form various enclosed structures, separate from the cytosol, which we refer to as organelles. These specialized compartments provide the environments required for specific biochemical reactions or the storage of proteins, small molecules or ions. A related form of compartmentalization is the separation of membranes into distinct domains, such as the apical and basolateral domains of the plasma membrane. The proper functioning of many cell types relies on the asymmetric specialization of these two membrane domains. In addition, the formation and proper orientation of an apico-basal polarity axis is essential for the building of multicellular tissues from individual cells. The underlying theoretical bedrock of the work presented in this thesis relies heavily on previous research relating to cell polarity, and an in-depth discussion on this topic is warranted. Therefore, **Chapter 2** of this thesis will provide a review of our current understanding of how cells establish and maintain an apical-basal polarity axis.

Maintaining the distinct membrane compartments depends on, incorporating into the membrane domains, the specific transmembrane proteins that make it unique. Vice versa, the correct functioning of many transmembrane-proteins (e.g. transporters or ion channels) is usually tied to their localization on a particular membrane domain. The mislocalization of such proteins to the wrong domain or to ectopic locations inside of the cell, can disrupt the capacity of a tissue to absorb and excrete metabolites (e.g. nutrient absorption in the intestine or excretion of biliary metabolites in the liver) or maintain the correct ion concentration gradient across the membrane. In this regard, it is not surprising that a multitude of diseases are associated with defects in polarity and the proper polarized trafficking of (transmembrane)proteins (Overeem et al., 2016; Stein et al., 2002; Treyer and Müsch, 2013; Wodarz and Näthke, 2007).

The primary focus of this thesis are two diseases in which defective protein transport to the apical membrane is the root cause of the symptoms of both diseases. These two diseases are microvillus inclusion disease (MVID) and Wilson disease. The majority of chapters are dedicated to improving our understanding of the

pathophysiological mechanisms underlying the development of MVID (Chapter 3-6). MVID is an autosomal recessive genetic disorder that is caused by mutations in the MYO5B gene, encoding the myosin Vb protein (Müller et al., 2008; Szperl et al., 2011). Myosin Vb is a motor protein that interacts with membrane vesicles through a specialized cargo-binding protein domain, and transports these vesicles by binding and detaching actin filaments in a processive manner (Velde et al., 2013). The cargo domain binds directly to the GTPases rab8 and rab11, which are bound to vesicles of recycling endosomes (albeit not exclusively in the case of rab8). Thus, myosin Vb is thought to primarily affect protein transport pathways that involve the recycling endosome.

The enterocytes of the intestine are most strongly affected by loss of myosin Vb. The trafficking of certain apical and basolateral proteins is mistargeted in myosin Vb deficient enterocytes (Overeem et al., 2016). Transmission electron microscopy of MVID intestinal samples reveals atrophy of the microvilli and the presence of cytoplasmic vacuoles lined with microvilli, which are referred to as microvillus inclusions. At the multicellular level, the small intestinal mucosa of patients show a variable degree of villus atrophy. These intestinal defects result in the main symptom of the disease: intractable chronic diarrhoea. The onset of this diarrhoea usually starts within the first days after birth, but in some cases it manifests later at around 3-4 months. Currently, an intestinal transplantation is the only viable treatment option. Without transplantation, patients are completely dependent on parental nutrition, which can cause severe complications over prolonged periods (e.g. sepsis). As a result, children suffering from this terrible disease have a short life expectancy, and better treatment options are direly needed. An increased understanding of this disease will be required to devise new treatment strategies. A more in-depth review of our current understanding of the disease mechanism of MVID, and several related chronic diarrheal disorders, is provided in **Chapter 3**.

The type and severity of symptoms can vary greatly between individual MVID patients (Dhekne et al., 2018; Halac et al., 2011). Differences in the type of myosin Vb mutation are a potential cause, but it is hard to confirm such genotype-phenotype correlations by examining patient samples alone, as there are multiple confounding variables that could affect the disease outcome. First, treatment given to MVID patients can be highly iatrogenic (e.g. liver cholestasis caused by parental

nutrition). Second, it can be hard to differentiate secondary effects of the disease from the ones directly caused by myosin Vb mutations. For example, patients do not eat, which is likely to have profound effects on the composition of the microbiota in the gut, which in turn could affect the cells of the intestinal tract. Third, it is difficult to exclude the influence of genetic variation between patients, which might sensitize or desensitize patients for the symptoms caused by MVID.

Chapter 4 describes the generation and characterization of a new MVID disease model through knock-out of the MyoVb gene in mice. This new model allows us to overcome some of the previously raised variability issues in studying the effect of Myosin Vb deficiency. MyoVb KO mice express no mutant protein, and any symptoms can be directly attributed to myosin Vb deficiency. Such a baseline understanding of what happens in the case of complete deficiency is important to interpret the possible effects of mutant myosin Vb variants that are expressed in MVID patients. In addition, a mouse model allows for examining relevant organ tissues postpartum or even earlier at an embryonic stage, removing the secondary disease effects and iatrogenic issues that affect human patient samples.

Additional methods are needed to understand the correlation between specific myosin Vb mutations and the outcome of the disease. An obvious is approach is to compile patient data and see if any correlations can be made between the type of mutations and the progression of the disease. Since MVID is such a rare disease, it is important to document and categorize data from as much patients as possible. Previously, our research group has documented all MVID patient mutations reported in literature in an online database (Velde et al., 2013). **Chapter 5** expands on this work, updating the database with all new patients reported since that time. We discuss the recent identification of two variant forms of MVID, which are caused by mutations in STX3 and STXBP2, rather than in MYO5B. Several *in vitro* studies have suggested a common disease mechanism that unifies these enteropathies (Knowles et al., 2015; Stepensky et al., 2013; Vogel et al., 2015; Weis et al., 2016; Wiegerinck et al., 2014). We provide new data from patient samples that supports this hypothesis. In addition, we discuss the recent identification of a group of patients suffering from intrahepatic cholestasis, which carry mutations in MYO5B, but without any of the conventional intestinal symptoms seen in MVID (Gonzales et al., 2017).

The aim of **Chapter 6** is to elucidate the link between myosin Vb and intrahepatic cholestasis. This link had been suspected for some time, as a significant amount of MVID patients (approximately 25%) develop cholestasis in addition to intestinal symptoms (Halac et al., 2011). In many cases, cholestasis specifically developed after receiving an intestinal transplant, suggesting the introduction of a functional bowel revealed a previously hidden cholestatic condition. However, it could not be ruled out that this cholestasis has iatrogenic origins, since parental nutrition is a known cause of cholestasis. Since then, the aforementioned discovery of a group of intrahepatic cholestasis patients carrying MYO5B mutations without conventional MVID symptoms, confirmed the causal link between myosin Vb deficiency and cholestasis. It is unlear why cholestasis only occurs in a subset of individuals carrying mutations in MYO5B, as is the mechanism through which myosin Vb deficiency disrupts bile acid metabolism.

Finding answers to these questions is important for deciding the correct course of action in treating MVID patients with organ transplantation. Patients who develop cholestasis specifically after a bowel transplantation, may need to have their transplant removed due to incompatibility of the treatment for cholestasis, with the immunosuppressive drugs that prevent transplant rejection. The solution for this is to perform a co-transplantation of both a bowel and a liver, for all patients. However, since not all patients develop cholestasis, a majority of patients would not have needed this liver in the first place. In such cases, the liver could have been used to treat a child suffering from another severe liver disease. Thus, if we know how patient mutations are related to cholestasis, we can prevent cholestasis from occurring following bowel transplantation, and make sure that only patients who need it receive a liver co-transplant.

From previous work, we know that overexpression of the cargo binding tail domain of myosin Vb in hepatic WIF-B9 cells, results in disrupted trafficking of bile acid transporters BSEP and ABBC2/MRP2 (Wakabayashi et al., 2005). This cargo domain-mutant is thought to act in a dominant negative manner, by competing with functional endogenous myosin Vb , and thereby disrupting myosin Vb mediated transport (Lapierre et al., 2001). In patient liver samples, ABCC2/MRP and BSEP were found to be mislocalized (Girard et al., 2014; Schlegel et al., 2018). These studies point towards a mechanism in which myosin vb deficiency disrupts the

delivery of important bile acid transporters to the plasma membrane. Much of the precise mechanism remains unclear however. In particular, these studies do not explain why cholestasis only occurs in a subset of patients. We investigate this issue in Chapter 6, where we employ multiple methods to interfere with myosin Vb function in hepatic cells. These methods include CRISPR-induced knock-out of MYO5B in the HepG2 hepatic cancer cell line, combined with overexpression of myosin Vb mutant variants. Moreover, several key findings from these HepG2 experiments are replicated in hepatocytes generated from pluripotent stem cells, and using MyoVb KO mice. The combination of these methods results in the most in depth analysis of the role of myosin Vb in hepatocytes yet, and provides a novel explanation for the variation in cholestasis development between MVID patients.

In the case of microvillus inclusion disease, symptoms are the result of the mislocalization of multiple proteins, due to a defect in the protein trafficking machinery. But the faulty delivery of a trans-membrane protein can also occur more directly, when a mutation in that protein directly affects its ability to be trafficked. This is the case with certain patients suffering from Wilson disease (WD), which is the focus of **Chapter 7** of this thesis. Wilson disease is an autosomal recessive disorder caused by mutation in the ATP7B gene, encoding the copper transporter protein ATP7B (Członkowska et al., 2018). In the liver, ATP7B is responsible for transporting copper taken up by hepatocytes from the bloodstream, into the bile canaliculi, thereby reducing the concentration of copper in the body. This process is disrupted in WD patients, which leads to accumulation of copper in the liver, and eventually also in other organs such as brain, kidneys and cornea. Accumulated copper in these organs then gives rise to a wide variety of symptoms, primarily hepatic and neurological. The symptoms of liver disease can vary greatly between patients, ranging from asymptomatic to acute liver failure. Similarly, neurological and psychiatric symptoms are variable, and can include: movement disorders (e.g. tremor), dystonia, headaches, insomnia and depression. Neurological symptoms typically manifest later than hepatic ones, and can occur without apparent hepatic symptoms. In the past, Wilson disease was fatal in all cases, leading inevitably to liver failure. Since then, great progression has been made in the pharmacological treatment of this disease through administration of copper chelating compounds. Treatment now typically consists of lifelong pharmacological treatment. Liver transplantation is reserved for severe or resistant cases.

ATP7B normally resides in the Golgi apparatus of hepatocytes, and is transported to the apical domain (which forms the bile canaliculi) in response to an increased concentration of copper in the body (Polishchuk et al., 2014). It was reported that a number of patient ATP7B mutations result in the ectopic localization of ATP7B in the endoplasmatic reticulum, including the H1069Q mutation, which is the most frequent mutation in the European and North American WD patient population (Payne et al., 1998). Interestingly, the H1069Q mutant retains (partial) functionality (van den Berghe et al., 2009; Iida et al., 1998; Payne et al., 1998), and thus it is thought that ATP7B mislocalization, not a lack of functional ATP7B, is the cause of the disease . Strategies to restore mutant ATP7B to the plasma membrane have shown some promising results (van den Berghe et al., 2009; Chesi et al., 2016). Compounds which aid protein folding, or prevent protein degradation, were capable of improving the copper-induced transport of ATP7B to bile canaliculi. These studies have been done using artificial overexpression of ATP7B mutants however, and further investigation using methods that more closely resemble the *in vivo* situation are necessary. In **Chapter 8**, we describe the development of a new WD model, by *in vitro* differentiation of induced pluripotent stemcells derived from WD patients to hepatocytes. For this purpose we first characterize the capability of human iPS derived hepatocytes (hiHeps) to polarize and form bile canaliculi *in vitro*, which is essential to study the polarized trafficking of ATP7B. This capability has been an overlooked aspect in the field, and our work provides the first extensive characterization of hiHep polarization. Using this novel model, we discover novel insights on the trafficking deficiencies of the mutant H1069Q-ATP7B protein.

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